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Characterization and regulation mechanism analysis of ubiquitin-conjugating family genes in strawberry reveals a potential role in fruit ripening

Mengyao Li^{1†}, Liangxin Wang^{1†}, Yiting Liu^{1†}, Yuanxiu Lin^{1,2}, Yunting Zhang^{1,2}, Yu Long¹, Chuanying Luo¹, Yong Zhang¹, Qing Chen¹, Pinwen Chen³, Yan Wang^{1,2}, Xiaorong Wang^{1,2}, Haoru Tang^{1,2} and Ya Luo^{1*}

Abstract

Background: E2 ubiquitin-conjugating (UBC) enzymes are an integral component of the ubiquitin proteasome system that play an important role in plant development, growth, and external stress responses. Several UBC genes have been identified in various plants. However, no studies exploring the functions of UBC genes in regulating fruit of strawberry have been reported. In the present study, a systematic analysis of the entire UBC family members were conducted in the genome of strawberry (*Fragaria × ananassa*) based on bioinformatics method, and the gene functioning in strawberry ripening was explored.

Results: A total of 191 UBC genes were identified in the genome of cultivated strawberry. These genes were unevenly distributed across the 28 chromosomes from the 4 subgenomes of cultivated strawberry, ranging from 3 to 11 genes per chromosome. Moreover, the expansion of FaUBC genes in strawberry was mainly driven by WGD. All the FaUBC genes were clarified into 13 groups and most of them were included in the group VI. The gene structure analysis showed that the number of exons varied from 1 to 23, and the structure of genes had few differences within the same groups but a distinction in different groups. Identification of the cis-acting elements of the promoter revealed multiple regulatory elements that responded to plant growth and development, phytohormone responsive, and abiotic and biotic stress. Data from functional annotation indicated that FaUBC genes play a role in a variety of biological processes. The RNA-seq data showed that FaUBC genes displayed different expression pattern during the fruit ripening process and clarified into 6 clusters. In particular, cluster 3 exhibiting a sudden expression increase in the turning red stage were speculated to be involved in fruit ripening. Hence, two FaUBC genes (*FaUBC76* and *FaUBC78*) were selected for gene function analysis by transient over-expression method. The results indicated that *FaUBC76* has a positive effect on the fruit development and ripening in strawberry by up-regulating accumulation of anthocyanins. Moreover, expression of some maturity-related genes were also significantly increased, further supporting a role for *FaUBC76* in the regulation of fruit ripening or softening. On the contrary, the overexpression of *FaUBC78* significantly

*Correspondence: luoya945@163.com

[†]Mengyao Li, Liangxin Wang and Yiting Liu contributed equally to this work.

¹ College of Horticulture, Sichuan Agricultural University, Chengdu 611130, China

Full list of author information is available at the end of the article



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increased the firmness of strawberry fruit, indicating that *FaUBC78* had a positive role in inhibiting the decrease of strawberry fruit firmness.

Conclusion: Our study not only provide comprehensive information on system evolution and function on UBC genes, but also give a new insight into explore the roles of FaUBC genes in the regulation of strawberry ripening.

Keywords: Strawberry, Ubiquitin-conjugating enzymes, Phylogenetic analysis, Expression patterns analysis, Fruit ripening

Introduction

As a ubiquitous post-translational modification for eukaryotes, ubiquitination is involved in many cellular processes, including hormone signaling transduction [1], apoptosis [2], and biotic and abiotic stresses stresses [3]. Protein ubiquitination requires the concerted action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). In the initial step, the E1 transfers ubiquitin (Ub) to the active site cysteine of E2 in an ATP-dependent manner and forms an E2-Ub intermediate. Subsequently, the E2-Ub conjugate interacts with an E3 to ligate Ub to a lysine side chain of the target protein and build mono- or poly-ubiquitin chains, then the target protein is degraded or modified to perform different functions [4].

The E2 genes exist as a multi-gene family in higher plants, which have been identified in rice [5], tomato [6], banana [7] and potato [8]. Being a key enzyme of ubiquitination process, E2 protein has a conserved UBC domain containing 150-200 amino acids in length [9]. Ubiquitin-conjugating enzyme plays a crucial role in plant growth and development [6], and also participates in environmental stresses [10, 11], immune response [12, 13], DNA damage and repair [14]. In Arabidopsis, *AtUBC32* is an endoplasmic reticulum-associated degradation (ERAD) component that functions in brassinosteroid-mediated salt stress tolerance [15]; *AtUBC2* is implicated in repression of flowering [16]; *AtUBC13* was involved in epidermal cell differentiation and iron deficiency responses [17, 18]. A tomato UBC13-type homologous protein, FNI3, is involved in the regulation of the immune response [19]. The expression levels of *Cucumis melo* UBC enzyme (*CmUBC*) were increased under drought and salt stress in melon [20]. Wheat defense systems against *Zymoseptoria tritici* can be regulated by *Triticum aestivum* ubiquitin-conjugating enzymes 4 (*TaU4*) [21]. The Arabidopsis *UBC22*-knockout mutants reduced the length and seed number of siliques, and caused an early arrest of nearly 90% ovules development [22]. It was reported that the regulator RIN (Ripening-inhibitor) of tomato fruit ripening could directly combine with the E2 promoter region, and the fruit color was changed after silencing the E2 gene [6].

Strawberry is a nutritionally important fruit as well as an ideal model plant for studying non-climacteric fruits. There is a growing recognition that fruit ripening regulation can meet the supply-demand balances of market to some extent. To regulate strawberry fruit development and ripening, previous researchers have mainly focused on abscisic acid (ABA) [23], auxin [24, 25] and sucrose [26]. Genome-wide analysis of the UBC genes in cultivated strawberry would be necessary for strawberry fruit development and ripening research. In this study, we performed a genome-wide analysis of UBC genes in strawberry with a focus on gene structure, evolutionary analysis, and expression abundance. Moreover, the role of E2 genes on strawberry fruit development and ripening were analyzed.

Studies have shown that ABA is a key hormone in the regulation of fruit ripening of strawberry and other non-climacteric fruit. ABA can promote the expression of genes related to strawberry fruit ripening [27]. At the same time, ABA can also promote the expression of other genes related to coloring and softening of non-respiratory climacteric fruits, such as chalcone synthase (*CHI*) and expansin (*EXP*) genes [28]. Moreover, the reception and transduction of ABA signals are regulated by protein ubiquitin modification [29, 30]. It reported that the *ubc32* mutant of E2 protein gene showed an insensitive phenotype to ABA during and after seed germination, and *UBC32* was a positive regulator of ABA signal [31]. Current studies have confirmed that ABA plays an important role in strawberry fruit ripening. ABA is closely related to ubiquitination, whereas ubiquitin modification plays a role in strawberry fruit ripening has not been reported. In view of this, this study intends to explore the function of E2 in strawberry fruit ripening, in order to enrich the regulatory network of strawberry fruit ripening.

Results

Genome-wide identification, and characteristics of FaUBC genes in strawberry

Based on the genome searching and domain confirmation, a total of 191 FaUBC genes were identified in the genome of cultivated strawberry. All the identified FaUBC genes were renamed according to the order of their chromosome location (Fig. 1). As the result

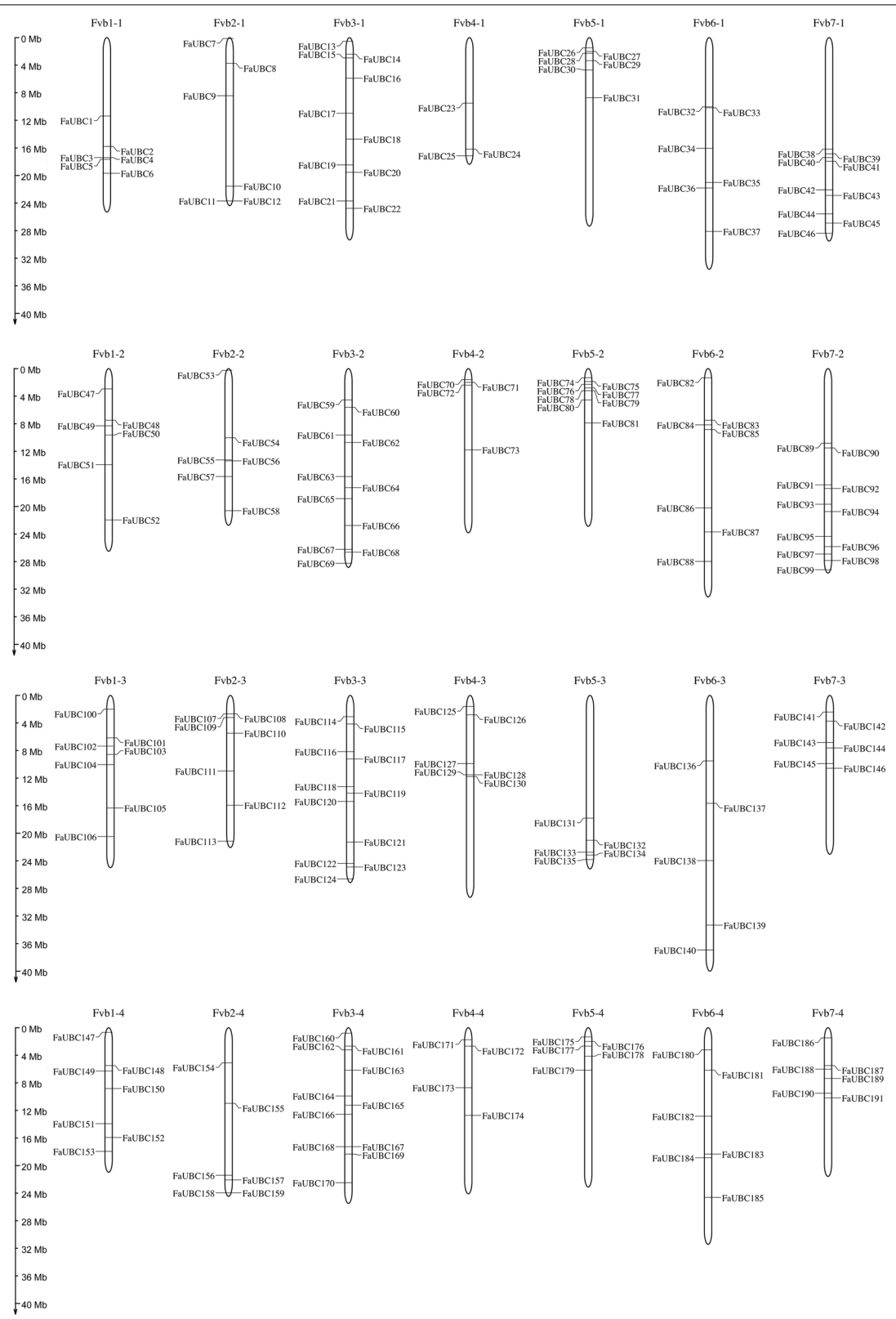


Fig. 1 Chromosomal distribution of UBC genes in the strawberry genome. Chromosome numbers are provided at the top of each chromosome. Scale is in megabases (Mb)

showed, 191 FaUBC genes were unevenly distributed across the 28 chromosomes in the 4 subgenomes of cultivated strawberry, ranging from 3 to 11 genes per chromosome. A maximum 11 FaUBC genes was located on chromosome 7 and 3 from the second subgenome (Fvb7-2 and Fvb3-2), as well as the chromosome 3 from the third and fourth subgenomes (Fvb3-3 and Fvb3-4), followed by 10 genes on chromosome 3 from the first subgenome (Fvb3-1). On the contrary, the minimum number of FaUBC genes (3 members) was distributed on chromosome 4 from the first subgenome (Fvb4-1).

The physicochemical properties of the 191 FaUBC proteins were also analyzed and shown in Table S1. The number of amino acids varied from 67 to 1921, and most of them (145 out of 191, 75.9%) were from 100 to 300. The protein molecular weights (MW) were from 7.478 to 216.883 KDa, and the isoelectric points (pI) were concentrated from 3.88 to 10.55. Except for FaUBC134 and FaUBC106, all of the other FaUBC genes didn't contain a signal peptide, which is essential for secreted proteins. Most of the FaUBC genes were predicted to be located in the cytoplasmic, chloroplast and nuclear, also some FaUBC genes were involved in mitochondrial, endoplasmic reticulum, plasma, vacuole, peroxisome and extracellular. Notably, there are some FaUBC genes were predicted to be dual-located, for instance, *FaUBC35* and *FaUBC139* were located in cytoplasmic and peroxisome, *FaUBC125* was located in both nuclear and cytoplasmic, while *FaUBC134* was located in extracellular, vacuole or endoplasmic reticulum.

Classification and structural analysis of FaUBC proteins in strawberry

According to the classification of UBCs in Arabidopsis, all the FaUBC proteins were classified into 13 groups (Fig. 2), most (37 members) of which were included in the group VI, followed by group IV (32 members). The gene structure was also investigated (Fig. S1): the number of exons varied from 1 to 23, and the structure of genes had few differences within the same groups but a distinction in different groups. In addition, the motif analysis showed that 10 motifs were found in the amino acids sequences of FaUBC proteins (Fig. S1). Motif 1 is Ubiquitin-conjugating domain and found in all of the FaUBC sequences. Motif 10 is specific in group XIV, while motif 9 encoding an Ubiquitin-conjugating domain and motif 8 is specific in group XI. The motifs 5 and 2 were also detected as Ubiquitin-conjugating domain and found in most of the FaUBC sequences.

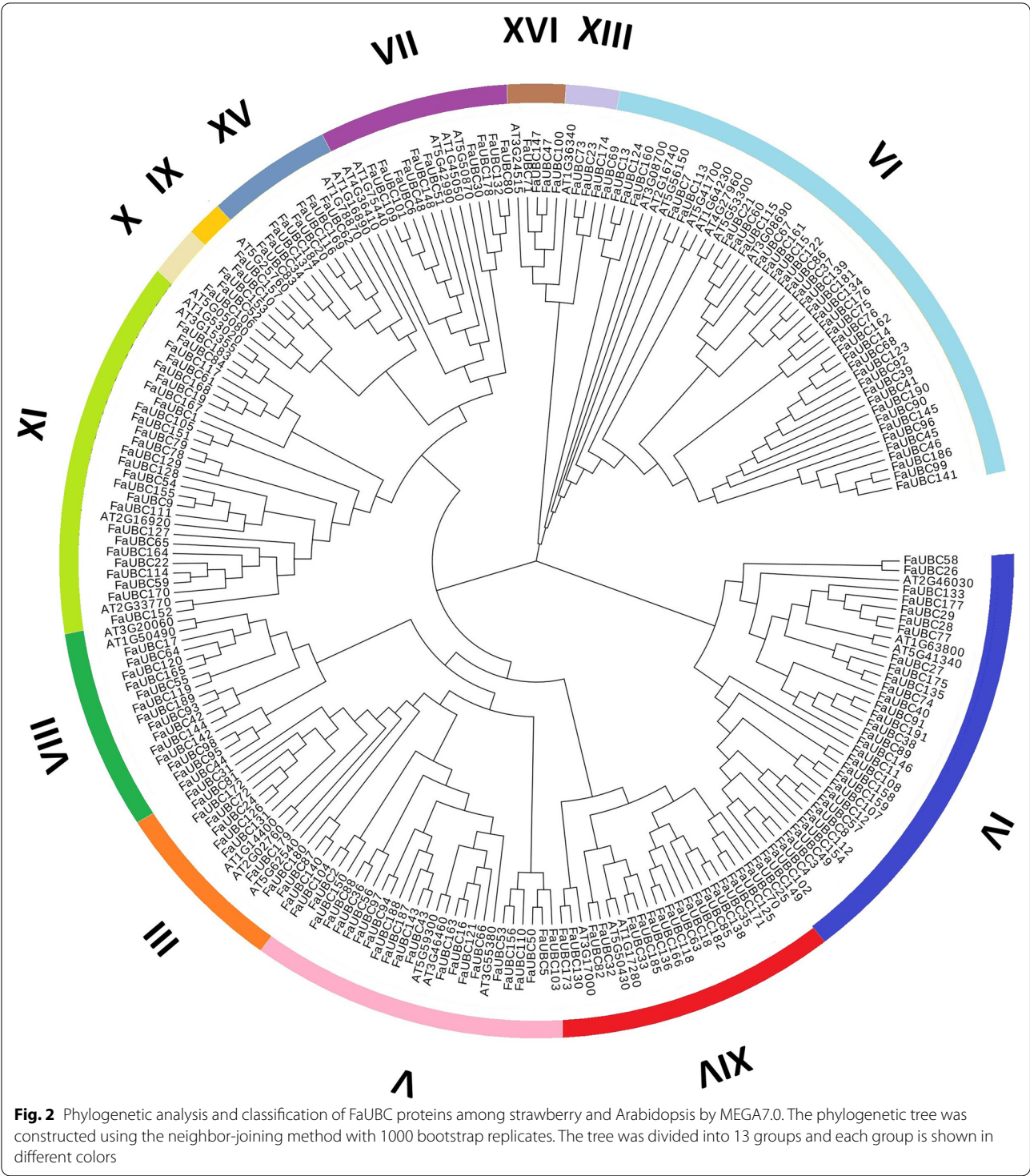
Synteny and evolutionary analysis of FaUBC genes in strawberry

The synteny relationship between Arabidopsis AtUBC genes and strawberry FaUBC genes was investigated to explore the origin and evolutionary process (Fig. 3). A total of 233 pairs of UBC genes were identified as collinear pairs in strawberry, while 78 collinear pairs were identified between Arabidopsis and strawberry (Table S2). Subsequently, the origins of duplication events of FaUBC genes in strawberry were detected using MCScanX package. The result showed that five types of duplication events were detected, including whole genome duplication (WGD) or segmental (WGD/segmental), Dispersed, Tandem, Singleton and Proximal duplication (Table S1). Most of which were WGD/segmental, followed by Dispersed with a number of 16 genes. Only *FaUBC3* and *FaUBC4*, *FaUBC128* and *FaUBC129* were duplicated from Tandem duplication. In addition, *FaUBC58* was detected in Singleton duplication event, while *FaUBC78*, *FaUBC79* and *FaUBC168* were duplicated from Proximal. These results suggested that the expansion of FaUBC genes in strawberry was mainly driven by WGD. Additionally, the number of non-synonymous substitutions per non-synonymous sites (Ka), the number of synonymous substitutions per synonymous sites (Ks) and the Ka/Ks ratio for paralogous gene pairs of FaUBC genes were calculated (Table S3). The results showed that the Ka and Ks ranged from 0 to 4.1 and 0.006–3.617 for identified paralogs respectively, the ratio of Ka/Ks ranged from 0 to 2.09. Most of the protein-coding genes have a Ka/Ks ratio of less than one, and only 23 gene pairs exhibited positive selection with a Ka/Ks ratio greater than one.

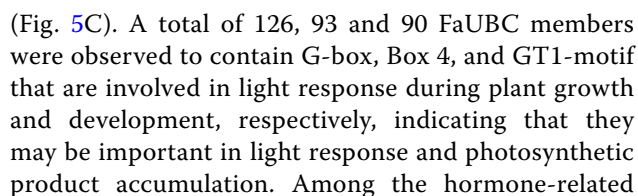
To illustrate the evolutionary relationship of UBC genes, a collinearity analysis of UBC genes among peach (*Prunus persica*), strawberry (*Fragaria × ananassa*), and pear (*Pyrus communis*) was constructed. The result revealed 69 and 74 UBC paralogous gene pairs in strawberry/peach and strawberry/pear, respectively (Fig. 4), and the ratio of Ka/Ks for each gene pair was analyzed (Table S4). According to the value of Ka and Ks, all the Ka/Ks ratios were lower than one, indicating that these paralogous gene pairs were under a strong purifying selection during evolution.

Analysis of the cis-acting elements of the FaUBC genes

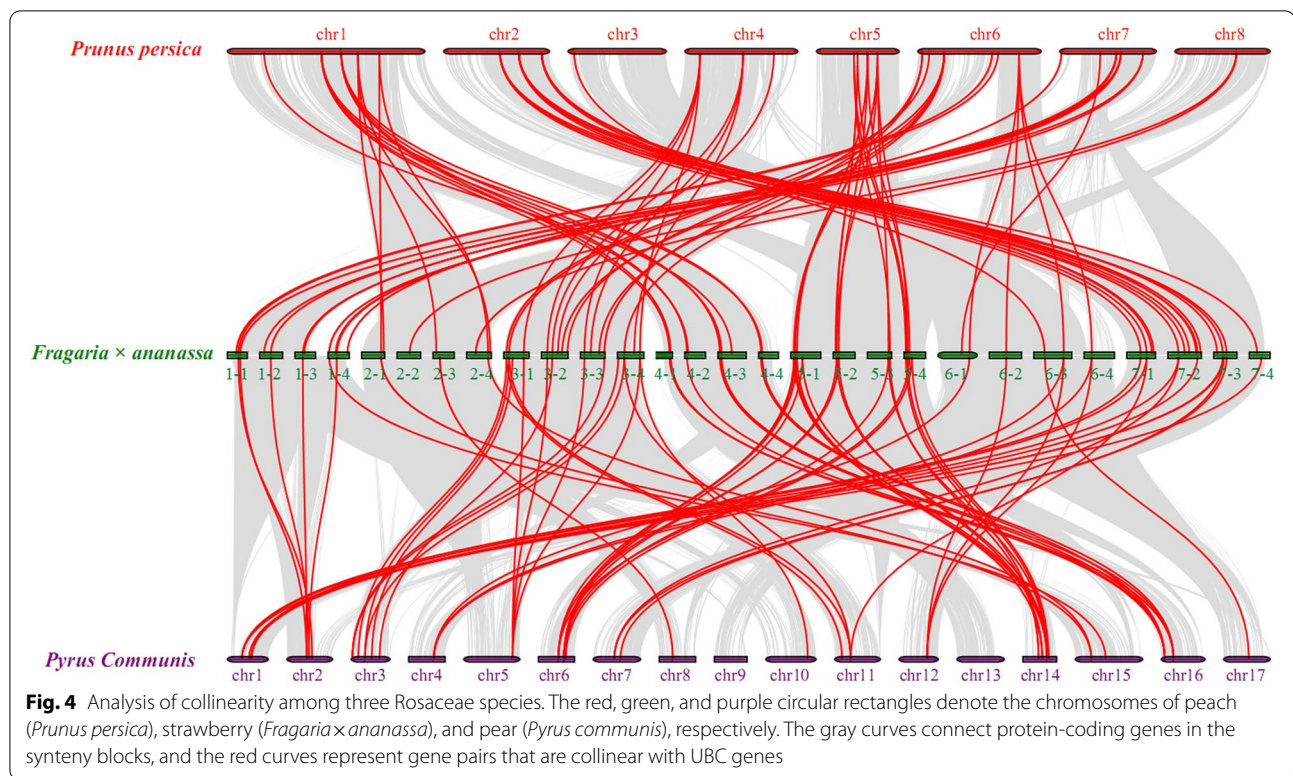
To further predict the function of the FaUBC genes, the cis regulatory elements in the UBC promoter regions were analyzed. The results showed that in addition to the basic core sequences TATA-box and CAAT-box which are unique to higher plant promoters, a total of 4642 elements belong to 76 cis element types were



identified in 191 UBC genes (Table S5). Most genes contain 21-30 cis elements, among them, *FaUBC176* contains the largest number of elements, with 53 elements, followed by *FaUBC88*, *FaUBC29*, *FaUBC76*, and *FaUBC28* (Fig. 5A). The element types contained in each UBC promoter also vary greatly, most of which are in 9-21 types (Fig. 5B). Based on the functional annotation, all the elements were grouped into three major classes: plant growth and development, phytohormone responsive, and abiotic and biotic stress



elements, 239 ABA response elements (ABRE), 211 methyl jasmonate response elements (CGTCA-motif), and salicylic acid response elements (TCA-element) were found in multiple UBC genes. In addition, drought response elements (MYB and MYC), anaerobic induction (ARE), and stress response elements (STRE) were also detected in the promoters of multiple UBC genes.



Functional annotation of FaUBC genes

The GO functional analysis of all FaUBC genes yielded 161 GO function terms (p -value < 0.05), including 122 biological process (BP) entries, 10 cell component (CC) entries, and 29 molecular function (MF) entries (Table S6). The GO terms “catalytic activity” and “transferase activity” were described the greatest number of genes with 168 and 167 members, and “protein ubiquitination”, “protein modification by small protein conjugation”, “ubiquitin-like protein transferase activity”, “ubiquitin activating enzyme activity”, “ubiquitin-protein transferase activity” and “ubiquitin conjugating enzyme activity” were also significantly enriched (Fig. 6A and Table S6). KEGG enrichment analysis results showed that 177 FaUBC genes were significantly enriched to 8 KEGG pathways (p -value < 0.05) (Fig. 6B and Table S7). Among them, 155 and 149 FaUBC genes were enriched into “Ubiquitin system” and “Ubiquitin mediated proteolysis” pathways, respectively. These annotations suggested that FaUBC genes are involved in a variety of biological processes.

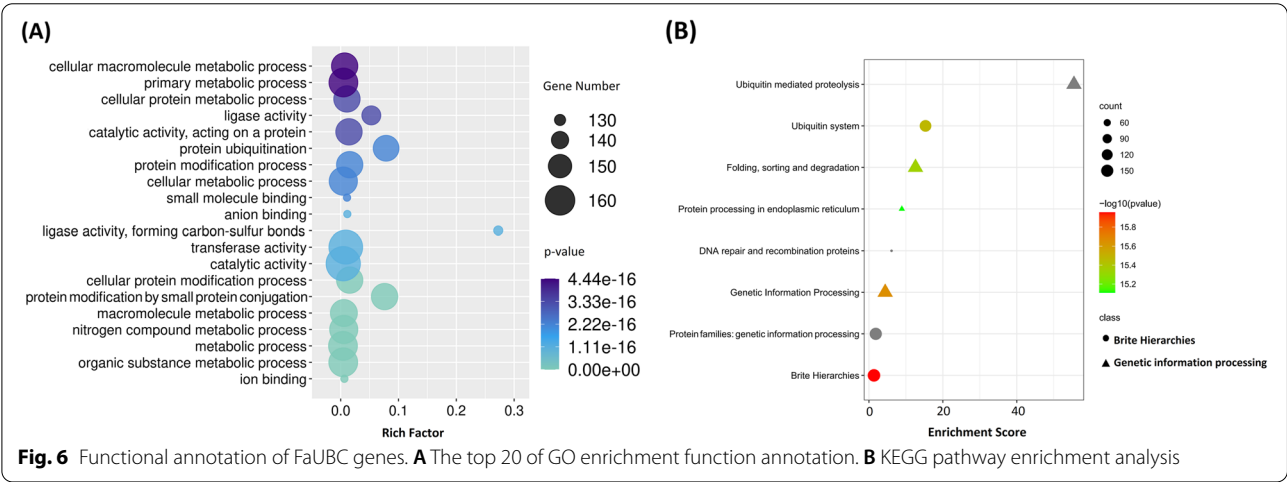
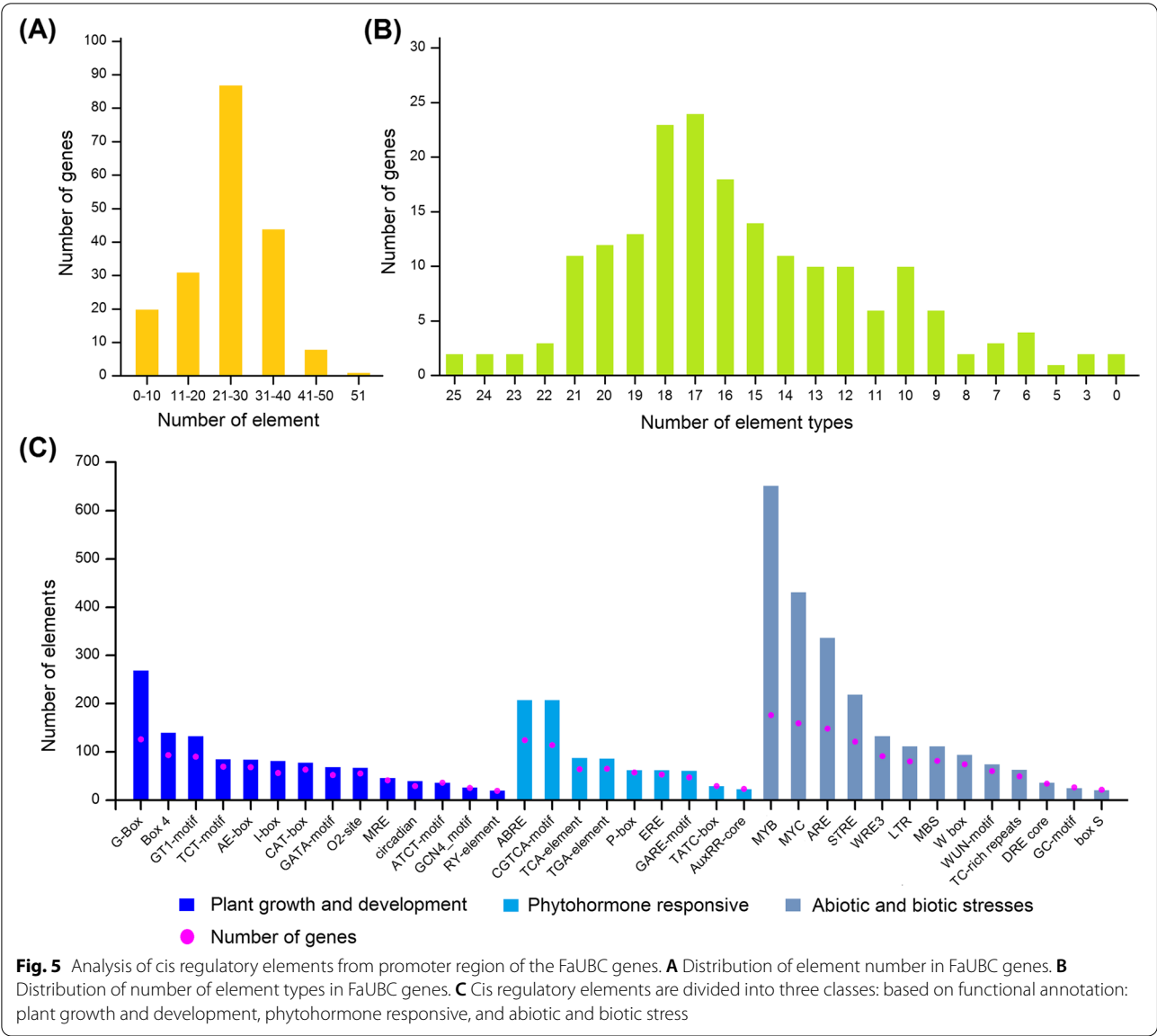
Protein-protein interaction network prediction of FaUBC proteins

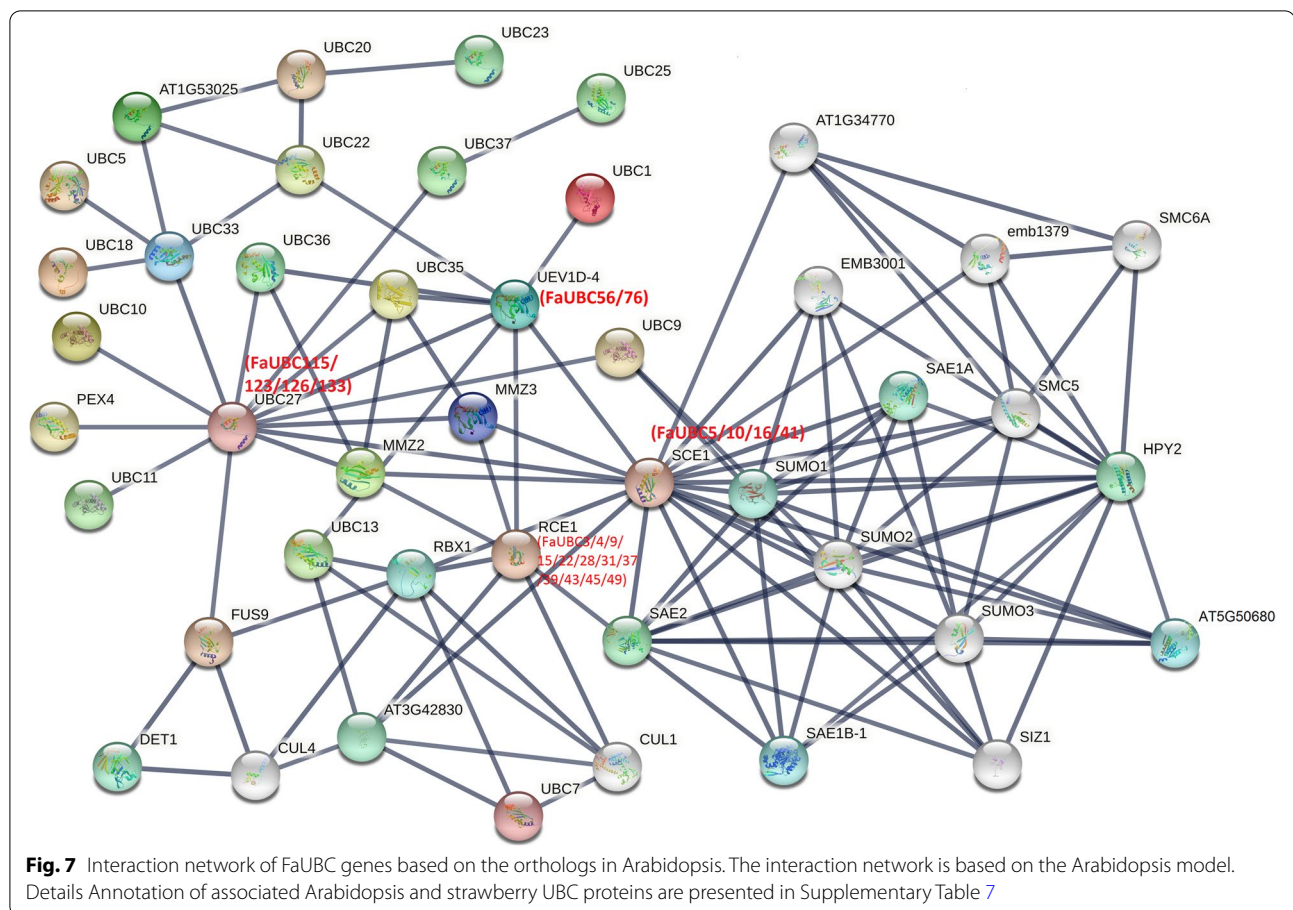
To predict the molecular interactions of FaUBC proteins, a protein-protein interaction analysis was performed

based on their orthologues AtUBC proteins in Arabidopsis. Of the 191 FaUBC proteins, 176 (92.1%) displayed homology to 28 AtUBC proteins from Arabidopsis (Table S8). As illustrated in Fig. 7, SCE1 (homolog of FaUBC5/10/16/41) showed interactions with 19 proteins, including several UBC proteins (such as UBC27 and UEVID-4) and other proteins in strawberry like RING-H2 protein (RBX1), SUMO-activating enzymes (SAE1B-1 and SAE2), and small ubiquitin-related modifier proteins (SUMO1, SUMO2 and SUMO3). It is also predicted that UBC27 (homolog of FaUBC115/123/126/133) can interact with 13 proteins. UEVID-4 (homolog of FaUBC56/76) also may interact directly with UBC proteins like UBC1, UBC13, UBC27, UBC35, and UBC36. In addition, RCE1 (homolog of FaUBC3/4/9/15/22/28/31/37/39/43/45/49) showed significant correlations with RBX1, SAE2 and CUL1 (Cullin protein).

Transcript abundance analysis of FaUBC genes in strawberry

To investigate the roles of FaUBC genes in the ripening of strawberry, the RNAseq-based expression was assessed. As illustrated in Fig. 8A, different FaUBC genes comprise different expression pattern during the fruit ripening process. Most genes were highly expressed in TR and FR stages. In general, according to their expression patterns, all the FaUBC genes could be clarified into 6 clusters as





shown in Fig. 8B. Among them, 14 genes belonged in cluster 2 showing a gradual increasing of expression during fruit ripening, and 41 members in cluster 3 exhibiting a sudden expression increase in the TR stage were speculated to be involved in fruit ripening. All the other genes involved in the other clusters were listed in Table S9.

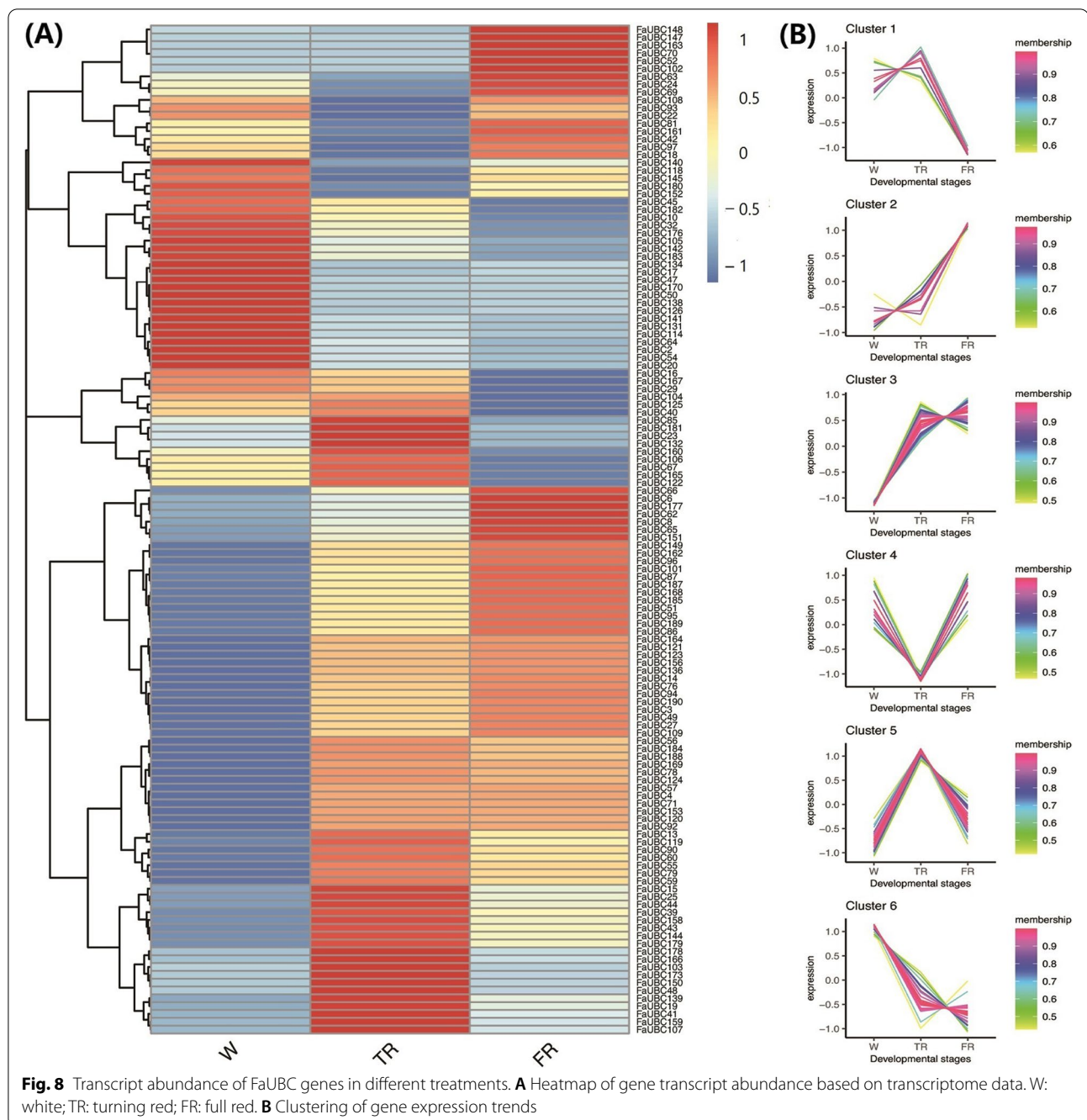
Validation of the function of *FaUBC76* and *FaUBC78* in strawberry fruit ripening

To validate the roles of *FaUBC* genes in strawberry fruit ripening, two *FaUBC* genes (*FaUBC76* and *FaUBC78*) included in the cluster 3 were selected for gene function analysis. A RT-qPCR analysis was performed to detect the UBC expression during fruit development stages. In agreement with the transcriptome data, the expression of *FaUBC76* and *FaUBC78* were abundantly expressed in FR, but at low levels in W stage (Figs. 9A and 10A). The two UBC genes were further validated by transient overexpression method.

At 5 days after injection, the fruit color of *FaUBC76* overexpression group was significantly redder than the control group (Fig. 9B). A significant higher expression level in the *FaUBC76* overexpression fruit than that of

the control was observed, indicating the transient overexpression successfully resulted in the up-regulation of *FaUBC76*. The firmness of *FaUBC76* overexpression fruit was lower than that of the control group (Fig. 9C), whereas the TA were not significant (Fig. 9D). In addition, the content of total anthocyanins in overexpression fruit was higher than that of the control group (Fig. 9E). Furthermore, RT-qPCR was used to measure the expression of a set of ripening-related genes, including ABA biosynthesis gene (*NCED1*), cell wall-related genes (cellulase 1/2, *CEL1/2*, and pectin lyase 1, *PL1*), anthocyanin biosynthesis related genes (chalcone isomerase, *CHI*; anthocyanidin synthase, *ANS* and *MYB10*). The results showed that *FaMYB10*, *FaPL1*, *FaCEL1/2* were up-regulated, and *FaNCED1*, *FaANS* and *FaCHI* had no significant difference compared with that of the control fruit (Fig. 9E). These results indicated that *FaUBC76* is a positive regulatory factor that promotes strawberry ripening.

Between CK and *FaUBC78* overexpression fruit groups, the fruits were harvest after 8 days when the CK turned to fully red first. The relative expression level of *FaUBC78* was significantly increased in the overexpression fruit (Fig. 10B). Notably, the overexpression of



FaUBC78 significantly increased the firmness of strawberry fruit, but had no significant effects on fruit TA and anthocyanin content (Fig. 10C~E). RT-qPCR was used to measure the transcript levels of a set of ripening-related genes, and the result showed that only the expression level of *FaCHI* was significant up-regulated in the *FaUBC78* overexpressed fruit (Fig. 10F). These work reveals *FaUBC78* play a role in inhibiting the

decrease of fruit firmness and delaying maturation time during fruit ripening.

Discussion

In this study, a total of 191 UBC genes were identified in the genome of *Fragaria × ananassa*. The number of FaUBC genes is far more than the 45, 48, 52, 75, and 74 UBC genes isolated from the genome of *Arabidopsis* [32],

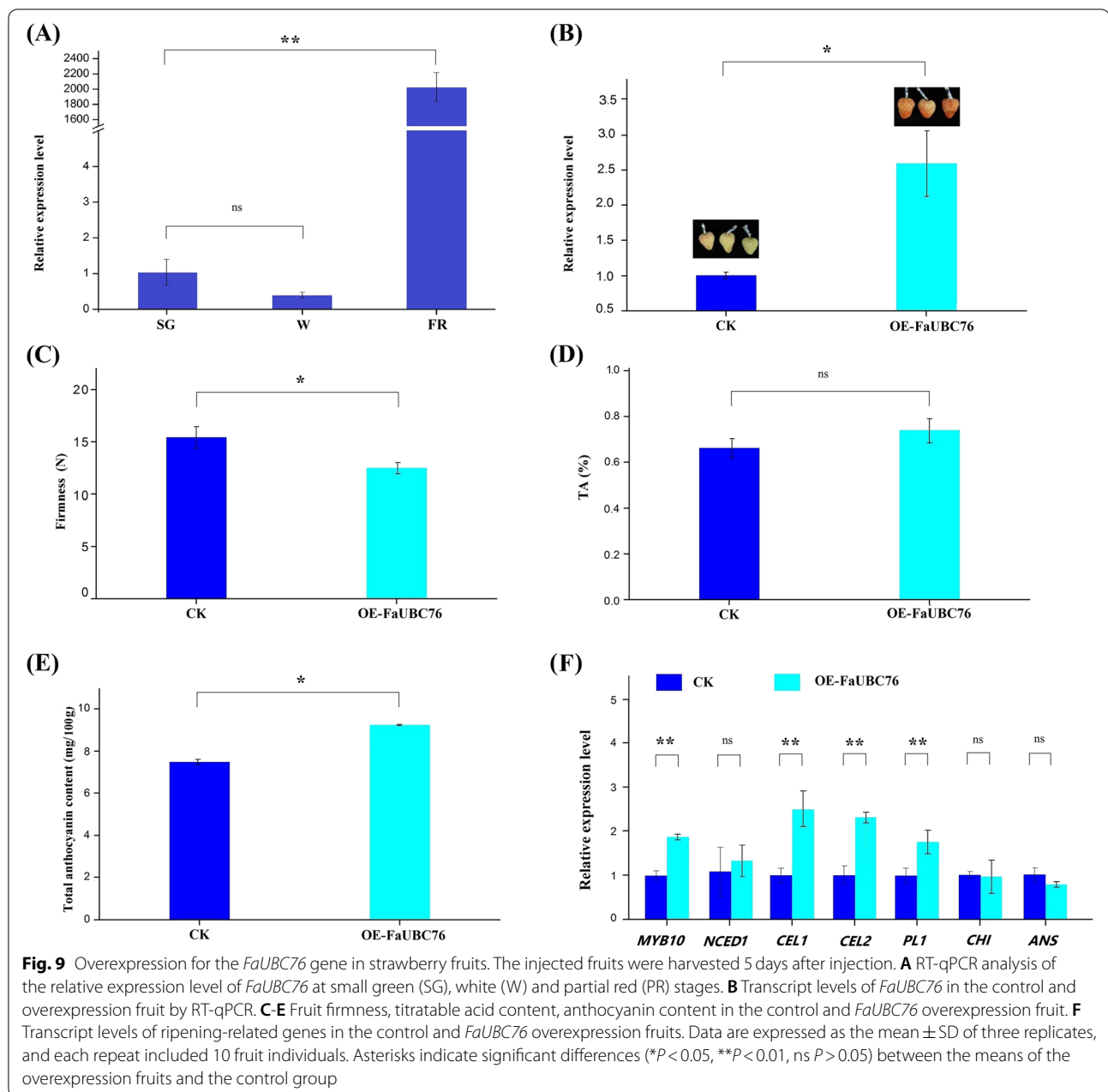
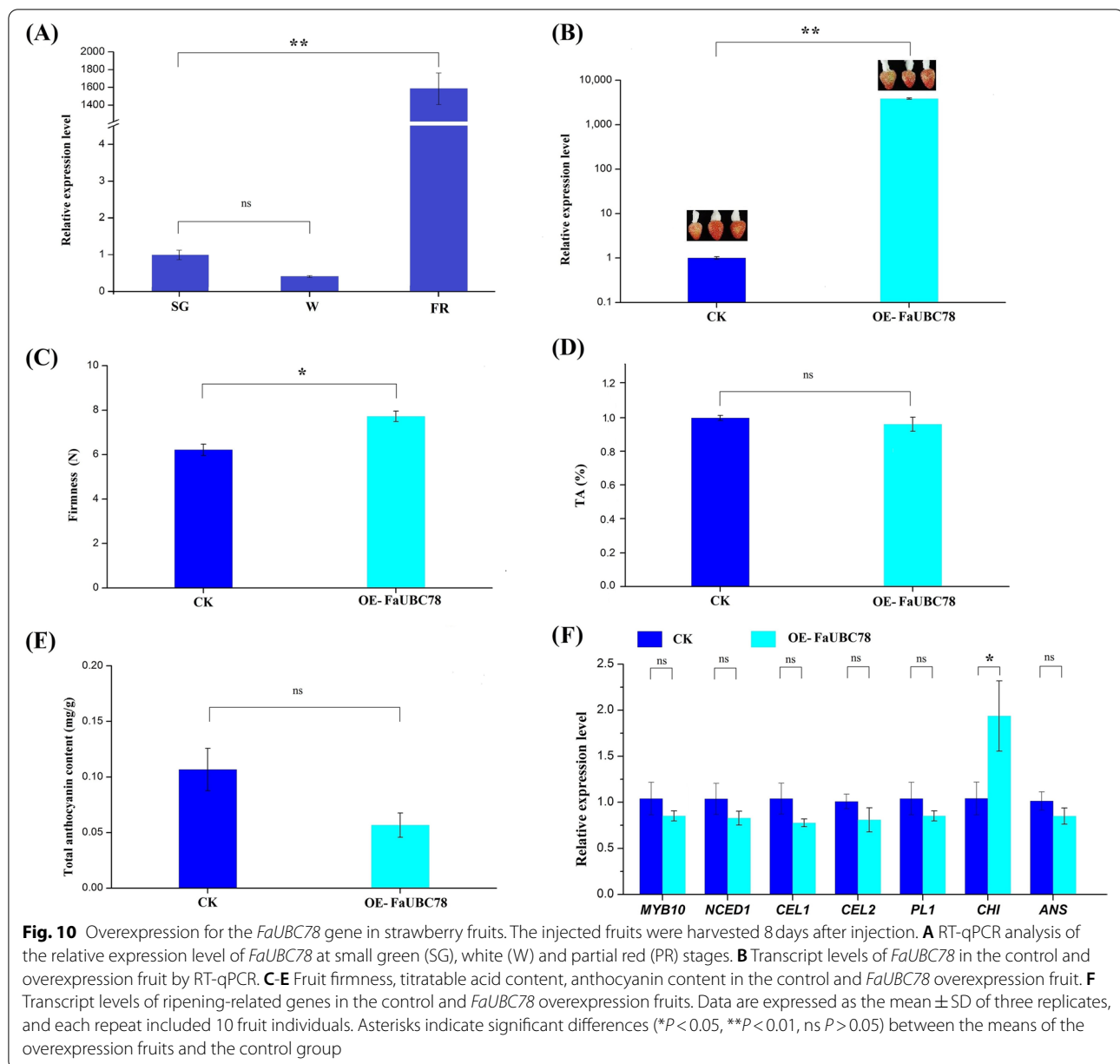


Fig. 9 Overexpression for the *FaUBC76* gene in strawberry fruits. The injected fruits were harvested 5 days after injection. **A** RT-qPCR analysis of the relative expression level of *FaUBC76* at small green (SG), white (W) and partial red (PR) stages. **B** Transcript levels of *FaUBC76* in the control and overexpression fruit by RT-qPCR. **C–E** Fruit firmness, titratable acid content, anthocyanin content in the control and *FaUBC76* overexpression fruit. **F** Transcript levels of ripening-related genes in the control and *FaUBC76* overexpression fruits. Data are expressed as the mean \pm SD of three replicates, and each repeat included 10 fruit individuals. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, ns $P > 0.05$) between the means of the overexpression fruits and the control group

rice [5], tomato [6], banana [7] and maize [33], which indicates that the UBC family expanded in strawberry. Gene duplication events such as whole genome duplication, tandem duplication, segmental duplication, and transposition have a remarkable role in the expansion of gene family members in genomes [34], and increasing evidences has shown that whole genome duplication are largely responsible for the expansion of gene families in cultivated strawberry, such as the GST, GMP and RALF gene families [35–37]. Additionally, the genome sizes of Arabidopsis, strawberry, rice, tomato, maize, and banana

are ~125, ~240, ~466, ~466, ~900, ~2300, and 523 Mb, respectively. Clearly, the higher number of UBC-encoding genes in strawberry can not only be explained by genome size.

Pear, peach, and strawberry are important fruit producing species belong to Rosaceae family, which share similarly genetic backgrounds. Previous studies also showed that there were more collinear regions in the genome, suggesting that the phylogenetic relationship of these three species was closely related [38]. In our study, a total of 69 and 74 UBC paralogous gene pairs



were revealed in strawberry/peach and strawberry/pear, respectively, supporting a strong evolutionary relationship of UBC genes in Rosaceae species. The Ka/Ks value can further explain the gene selection pressure and evolution rate positive selection: Ka/Ks > 1 suggested positive selection, Ka/Ks = 1 suggested neutral selection, and Ka/Ks < 1 suggested negative selection [39]. All paralogous genes in UBC gene family among strawberry/peach and strawberry/pear have experienced purifying selection with the ratio of Ka/Ks less than one. These findings of collinearity analysis indicate that the genes were retained in Rosaceae species after

whole genome duplication event and were strongly retained by stabilizing selection [40].

To further understand the regulatory network of UBC proteins, an interaction network was built. Most UBC proteins exhibited interactions with multiple proteins including several UBC proteins, RBX1, SUMO. A RING-H2 Protein, RBX1, bind the ubiquitin-conjugating enzyme E2 and bring it into close proximity with the E3 substrate [41]. And RCE1 (homolog of FaUBC3/4/9/15/22/28/31/37/39/43/45/49) which is interacts directly with RBX1 in Arabidopsis and play important role in early development [42]. There are other studies showing that

an E2 ubiquitin ligase UBC-9 mediates covalent attachment of small ubiquitin-related modifier (SUMO) [43, 44]. This provided a useful reference for in-depth understanding the molecular interactions of FaUBC proteins.

Despite the potential functional significance of UBC members, only a few UBC family members have been described in horticultural plants with fruits as product organs, such as tomato [6], banana [7], papaya [45], grape [46] and pear [47]. Among them, several studies have been demonstrated that E2s are involved in the fruit ripening process. *SlUBC32*, which is upregulated during tomato (*Solanum lycopersicum*) fruit ripening and downregulated in the *rin* mutant, plays an important role in the regulation of fruit ripening [6]. Some of MaUBC genes are up- and downregulated during different ripening stages in banana (*Musa acuminata*) [7]. Thirteen (*CpUBC4/6/7/8/9/11/12/14/16/19/20/28/34*) and two (*CpUBC2* and *CpUBC10*) of the 34 *CpUBC* genes in papaya (*Carica papaya*) were up- or downregulated during the progression of fruit ripening, respectively [45]. To investigate the roles of *FaUBC* genes in the ripening of strawberry, the expression pattern of *FaUBC* genes were analyzed during the fruit ripening process. The results showed that cluster 3 exhibiting a sudden expression increase in the turning red stage were speculated to be involved in fruit ripening. Subsequently, two *FaUBC* genes (*FaUBC76* and *FaUBC78*) included in the cluster 3 were randomly selected for gene function analysis by transient overexpression method. The results showed that *FaUBC76* overexpression gave rise to higher anthocyanin content and lower firmness than the control. It has been documented that the color, firmness and soluble solids of fruit are important signs of strawberry ripening. For most fruit trees, fruit ripening is usually characterized by fruit softening and color transformation, accompanied by complex physiological and biochemical changes and volatile metabolism to form unique quality [48]. Furthermore, the gene expression analysis showed that *FaUBC76* overexpression can significantly increase gene expression of *FaMYB10*, *FaPL1*, *FaNCED1* and *FaCEL1/2* that involved in strawberry ripening. Therefore, we speculate that *FaUBC76* has a positive effect on the fruit development and ripening in strawberry. *FaUBC78* overexpression significantly enhance the strawberry firmness, but had no significant effects on fruit TA and anthocyanin content. Moreover, only the expression level of *FaCHI* was significant up-regulated in the *FaUBC78* overexpressed fruit, while other ripening-related genes had no significant change. Taken together, these findings suggested that the strawberry UBC family genes might be participated in the regulation of fruit development and ripening processes. However, only two genes of UBC family was studied in this experiment, so the regulation of other genes of

the same family in strawberry fruit ripening needs to be further explored.

Materials and methods

Identification and comprehensive analysis of FaUBC genes

The UBC domain (PF00179) obtained from Pfam database was used as a query to search against the genome of strawberry downloaded from Genome Database for Rosaceae (GDR, <https://www.rosaceae.org>) [49]. The sequences with high score in the results were retrieved as putative UBC proteins. The number of amino acids, ORF length, putative protein molecular weights (MW) and isoelectric points (pI) for each sequence were obtained using a perl script. Multiple alignment was conducted using Geneious Prime software (<https://www.geneious.com/>). The conserved domains in the *FaUBC* proteins were screened and annotated based on Pfam [50], and NCBI-CDD [51]. The exon-intron structure of the *FaUBC* genes was analyzed using Gene Structure Display Server v.2.0 (<http://gsds.cbi.pku.edu.cn/index.php>). To identify the conserved motifs of *FaUBC* proteins, the MEME online program (<http://meme-suite.org/tools/meme>) was used with the following parameters: any number of repetition, maximum number of motifs was set as 10, and optimum motif length was set to 6-100 residues. The upstream 1500bp regions of *FaUBC* genes were downloaded from the genome data and were performed to analyze the cis regulatory element using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The interaction networks of the identified proteins were analyzed by STRING (<https://string-db.org/>). GO and KEGG functional annotation of *FaUBC* proteins were performed by PANNZER2 (<http://ekhidna2.biocenter.helsinki.fi/sanspanz/>) and DAVID (<https://david.ncifcrf.gov/>), respectively.

Phylogenetic and evolutionary analysis of UBC proteins

The Arabidopsis UBC protein sequences were downloaded from the Arabidopsis information source (TAIR) database, and the genomic data for pear (*Pyrus communis*) and peach (*Prunus persica*) were downloaded from Genome Database for Rosaceae (GDR, <https://www.rosaceae.org>). Phylogenetic tree of *FaUBC* proteins was constructed using Clustal X v.2.0 and MEGA v.7.0 software [52] with the neighbor-joining (NJ) method, and a bootstrap test with 1000 replicates were chose to evaluate the support of interior branches. The collinear gene pair was calculated using MCScanX software (<http://chibba.pgml.uga.edu/mcscan2/>), the syntenic relationship was visualized by Circos (<http://circos.ca/>). Synonymous (Ks) and non-synonymous (Ka) substitutions per site between duplicated *FaUBC* gene pairs were subsequently calculated using KaKs Calculator v.1.2 software [53].

Plant materials, RNA extraction, and cDNA synthesis

The strawberry (*Fragaria × ananassa* cv. Benihoppe) used as the experimental materials in our study were supplied by the Hanyuan County Dr. Luo Agriculture Co., Ltd. (Sichuan, China). The fruits at small green (SG), white (W) and full red (FR) stages were collected separately. These materials were quickly frozen and stored at -80 °C for subsequent experiments.

Total RNA was extracted from frozen strawberry fruit using the total RNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The RNA concentration was determined using a NanoDrop ND 2000 spectrophotometer, and purity was measured by the ratio OD260/OD280 (1.8–2.0) and OD260/OD230 (2.0–2.2). Finally, the integrity of the RNA was examined by 1.0% agarose gel electrophoresis. cDNA was synthesized from 1.0 µg of total RNA using the TransGene reverse transcription kits (Beijing, China). All cDNA samples were diluted 1:10 with RNase-free water for gene cloning and quantitative real-time PCR (RT-qPCR).

Transcript abundance analysis and RT-qPCR analysis of FaUBC genes

The transcript abundance levels of FaUBC genes in strawberry were retrieved from the online transcriptomic data (SRA accession: SRX6381727). The expression cluster analysis was analyzed by TCseq R package.

RT-qPCR analysis was carried out using SYBR Green Premix Ex Taq™ (Takara, Japan) on a CFX96 qPCR system (Bio-Rad, USA) in triplicate of each sample. The relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method [54]. *Fa26S* rRNA (accession: X58118) was used as the reference gene to standardize the raw data. All primers used in the present study were listed in Table S10.

Transient overexpression of *FaUBC76* and *FaUBC78* in strawberry fruit

Full-length coding sequence of *FaUBC76* and *FaUBC78* was amplified using the cDNA sample. Then these two genes were cloned into the modified pCAMBIA1301 vector with CaMV35s promoter, respectively. The recombinant vectors were then introduced into the *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* infiltration was performed based on the previously described method [55]. The agrobacterium suspension was injected in the entire fruits at white (W) stage when they were still attached to the plants by a sterile 1 mL syringe. The injected fruits were harvested when they turned fully red after injection. As control, fruits at the same stage were injected with bacteria containing empty vector. Each

treatment included three replicates, and each repeat included 10 fruit individuals.

Determination of the TSS, firmness, the TA and total anthocyanin content

The fruit firmness was measured by using a digital display firmness tester (FR-5105, LUTRON, China). The total titratable acid (TA) content was measured by titration with 0.1 M NaOH titration, and expressed as a citric acid content percentage. Anthocyanin content determination was carried out by the pH-differential method using buffer solutions of sodium acetate (0.4 M, pH 1.0) and potassium chloride (0.025 M, pH 4.5), and the absorbance was read at 520 and 700 nm, respectively [56].

Statistical analysis

The experiment was arranged in a completely randomized design with three replications. Statistical analysis was performed using multivariate logistic regression with the IBM SPSS statistics program (SPSS Version 27). Data were represented as average \pm STDEV ($n = 3$). Analysis of variance (ANOVA) was used to determine the significant difference when $p < 0.05$.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-021-03421-8>.

Additional file 1: Figure S1. Schematic gene structure and motif location of FaUBC genes.

Additional file 2: Table S1. The basic information of FaUBC genes in the genome of cultivated strawberry.

Additional file 3: Table S2. Collinear pairs identified between Arabidopsis and strawberry.

Additional file 4: Table S3. Ka, Ks and Ka/Ks of paralogous gene pairs of FaUBC genes.

Additional file 5: Table S4. Ka, Ks and Ka/Ks of paralogous gene pairs of UBC genes among three Rosaceae species.

Additional file 6: Table S5. Analysis of cis-regulatory elements in the promoter regions of FaUBC genes.

Additional file 7: Table S6. GO categories and distribution of FaUBC genes.

Additional file 8: Table S7. KEGG function classification of FaUBC genes.

Additional file 9: Table S8. The AtUBC proteins in protein-protein interaction network and the homologous FaUBC proteins in strawberry.

Additional file 10: Table S9. Expression of FaUBC genes and expression clusters.

Additional file 11: Table S10. Primers of ripening-related genes used to detect the expression level in overexpressed fruits.

Acknowledgements

Not Applicable.

Authors' contributions

M.Y.L., L.X.W., Y.T.L. and Y.L. (Ya Luo) initiated and designed the research. L.X.W., Y.T.L., Q.C., and Y.T.Z. performed the experiments. Y.X.L., Y.L. (Yu Long), Y.W., C.Y.L. and Y.Z. analyzed the data. M.Y.L. and L.X.W. wrote the paper. H.R.T., P.W.C. and X.R.W. revised the paper. Y.L. (Ya Luo) contributed reagents/materials/analysis tools. All authors have read and approved the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (3180817), the Key Projects of Sichuan Provincial Science and Technology Department (2018NZ0126), the Open Research Fund Program of Departmental and Municipal Coconstruction of Crops Genetic Improvement of Hill Land Key Laboratory of Sichuan Province (2021CGIHL05), the Cooperation Project of Ya'an City and Sichuan Agricultural University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials

The following information was supplied regarding data availability: RNA-Seq data are available at the NCBI Sequence Read Archive: SRX6381727. The genome sequence data is available at GDR database (GDR, <https://www.rosaceae.org>).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹College of Horticulture, Sichuan Agricultural University, Chengdu 611130, China. ²Institute of Pomology and Olericulture, Sichuan Agricultural University, Chengdu 611130, China. ³Departmental and Municipal Co-construction of Crops Genetic Improvement of Hill Land Key Laboratory of Sichuan, Nan-chong 637000, China.

Received: 20 October 2021 Accepted: 24 December 2021

Published online: 19 January 2022

References

- Santner A, Estelle M. The ubiquitin-proteasome system regulates plant hormone signaling. *Plant J*. 2010;61:1029–40. <https://doi.org/10.1111/j.1365-3113.2010.04112.x>.
- Hershko A, Ciechanover A. The ubiquitin system for protein degradation. *Annu Rev Biochem*. 1992;61:761–807. <https://doi.org/10.1146/annurev.bi.61.070192.003553>.
- Lee JH, Kim WT. Regulation of abiotic stress signal transduction by E3 ubiquitin ligases in Arabidopsis. *Mol Cells*. 2011;3:201–8. <https://doi.org/10.1007/s10059-011-0031-9>.
- Sharma B, Joshi D, Yadav PK, Gupta AK, Bhatt TK. Role of ubiquitin-mediated degradation system in plant biology. *Front Plant Sci*. 2016;7:806. <https://doi.org/10.3389/fpls.2016.00806>.
- Bae H, Kim WT. Classification and interaction modes of 40 rice E2 ubiquitin-conjugating enzymes with 17 rice ARM-U-box E3 ubiquitin ligases. *Biochem Biophys Res Commun*. 2014;444(4):575–80. <https://doi.org/10.1016/j.bbrc.2014.01.098>.
- Wang Y, Wang W, Cai J, Zhang Y, Qin G, Tian S. Tomato nuclear proteome reveals the involvement of specific E2 ubiquitin-conjugating enzymes in fruit ripening. *Genome Biol*. 2014;15:548. <https://doi.org/10.1186/s13059-014-0548-2>.
- Dong C, Hu H, Jue D, Zhao Q, Chen H, Xie J, et al. The banana E2 gene family: genomic identification, characterization, expression profiling analysis. *Plant Sci*. 2016;245:11–24. <https://doi.org/10.1016/j.plantsci.2016.01.003>.
- Liu WG, Tang X, Zhu X, Qi XH, Zhang N, Si HJ. Genome-wide identification and expression analysis of the E2 gene family in potato. *Mol Biol Rep*. 2019;46(1):777–91. <https://doi.org/10.1007/s11033-018-4533-9>.
- van Wijk SJ, Timmers HT. The family of ubiquitin-conjugating enzymes (e2s): deciding between life and death of proteins. *FASEB J*. 2010;24(4):981–93. <https://doi.org/10.1096/fj.09-136259>.
- Ahn MY, Oh TR, Seo DH, Kim JH, Cho NH, Kim WT. Arabidopsis group XIV ubiquitin-conjugating enzymes AtUBC32, AtUBC33, and AtUBC34 play negative roles in drought stress response. *J Plant Physiol*. 2018;230:73–9. <https://doi.org/10.1016/j.jplph.2018.08.010>.
- Jue D, Sang X, Liu L, Shu B, Wang Y, Xie J, et al. The ubiquitin-conjugating enzyme gene family in Longan (*Dimocarpus longan* Lour.): genome-wide identification and gene expression during flower induction and abiotic stress responses. *Molecules*. 2018;23(3):662. <https://doi.org/10.3390/molecules23030662>.
- Zhou B, Mural RV, Chen X, Oates ME, Connor RA, Martin GB, et al. A subset of ubiquitin-conjugating enzymes is essential for plant immunity. *Plant Physiol*. 2017;173(2):1371–90. <https://doi.org/10.1104/pp.16.01190>.
- Qin B. The function of Rad6 gene in *Hevea brasiliensis* extends beyond DNA repair. *Plant Physiol Biochem*. 2013;66:134–40. <https://doi.org/10.1016/j.plaphy.2013.02.013>.
- Tsui C, Raguraj A, Pickart CM. Ubiquitin binding site of the ubiquitin E2 variant (UEV) protein Mms2 is required for DNA damage tolerance in the yeast RAD6 pathway. *J Biol Chem*. 2005;280(20):19829–35. <https://doi.org/10.1074/jbc.M414060200>.
- Cui F, Liu L, Zhao Q, Zhang Z, Li Q, Lin B, et al. Arabidopsis ubiquitin conjugase UBC32 is an ERAD component that functions in brassinosteroid-mediated salt stress tolerance. *Plant Cell*. 2012;24(1):233–44. <https://doi.org/10.1105/tpc.111.093062>.
- Xu L, Ménard R, Berr A, Fuchs J, Cognat V, Meyer D, et al. The E2 ubiquitin-conjugating enzymes, AtUBC1 and AtUBC2, play redundant roles and are involved in activation of FLC expression and repression of flowering in Arabidopsis thaliana. *Plant J*. 2009;57:279–88. <https://doi.org/10.1111/j.1365-3113.2008.03684.x>.
- Wen R, Newton L, Li G, Wang H, Xiao W. Arabidopsis thaliana UBC13: implication of error-free DNA damage tolerance and Lys63-linked poly-ubiquitylation in plants. *Plant Mol Biol*. 2006;61:241–53. <https://doi.org/10.1007/s11103-006-0007-x>.
- Li W, Schmidt W. A lysine-63-linked ubiquitin chain-forming conjugase, UBC13, promotes the developmental responses to iron deficiency in Arabidopsis roots. *Plant J*. 2010;62:330–43. <https://doi.org/10.1111/j.1365-3113.2010.04150.x>.
- Mural RV, Liu Y, Rosebrock TR, Brady JJ, Hamera S, Connor RA, et al. The tomato Fni3 lysine-63-specific ubiquitin-conjugating enzyme and suv ubiquitin E2 variant positively regulate plant immunity. *Plant Cell*. 2013;25:3615–31. <https://doi.org/10.1105/tpc.113.117093>.
- Baloglu MC, Patir MG. Molecular characterization, 3D model analysis, and expression pattern of the CmUBC gene encoding the melon ubiquitin-conjugating enzyme under drought and salt stress conditions. *Biochem Genet*. 2014;52(1–2):90–105. <https://doi.org/10.1007/s10528-013-9630-9>.
- Millyard L, Lee J, Zhang C, Yates G, Sadanandom A. The ubiquitin conjugating enzyme, Ta U4 regulates wheat defence against the phytopathogen *Zymoseptoria tritici*. *Sci Rep*. 2016;6:35683. <https://doi.org/10.1038/srep35683>.
- Wang S, Cao L, Wang H. Arabidopsis ubiquitin-conjugating enzyme UBC22 is required for female gametophyte development and likely involved in Lys11-linked ubiquitylation. *J Exp Bot*. 2016;67(11):3277–88. <https://doi.org/10.1093/jxb/erw142>.
- Li C, Jia H, Chai Y, Shen Y. Abscissic acid perception and signaling transduction in strawberry: a model for non-climacteric fruit ripening. *Plant Signal Behav*. 2011;6(12):1950–3. <https://doi.org/10.4161/psb.6.12.18024>.
- Mezzetti B, Landi L, Pandolfini T, Spena A. The defH9-iaaM auxin-synthesizing gene increases plant fecundity and fruit production in strawberry and raspberry. *BMC Biotechnol*. 2004;4:4. <https://doi.org/10.1186/1472-6750-4-4>.
- Narayanan KR, Mudge KW, Poovaiah BW. Demonstration of auxin binding to strawberry fruit membranes. *Plant Physiol*. 1981;68(6):1289–93. <https://doi.org/10.1104/pp.68.6.1289>.

26. Jia HF, Chai YM, Li CL, Lu D, Luo JJ, Qin L, et al. Absciscic acid plays an important role in the regulation of strawberry fruit ripening. *Plant Physiol.* 2011;157(1):188–99. <https://doi.org/10.1104/pp.111.177311>.
27. Stone SL, Williams LA, Farmer LM, Vierstra RD, Callis J. KEEP ON GOING, a RING E3 ligase essential for Arabidopsis growth and development, is involved in abscisic acid signaling. *Plant Cell.* 2006;18(12):3415–28. <https://doi.org/10.1105/tpc.106.046532>.
28. Guo J, Wang S, Yu X, Dong R, Li Y, Mei X, et al. Polyamines regulate strawberry fruit ripening by ABA, IAA, and ethylene. *Plant Physiol.* 2018;177(1):339–51. <https://doi.org/10.1104/pp.18.00245>.
29. Jia H, Jiu S, Zhang C, Wang C, Tariq P, Liu Z, et al. Absciscic acid and sucrose regulate tomato and strawberry fruit ripening through the abscisic acid-stress-ripening transcription factor. *Plant Biotechnol J.* 2016;14(10):2045–65. <https://doi.org/10.1111/pbi.12563>.
30. Sutter JU, Sieben C, Hartel A, Eisenach C, Thiel G, Blatt MR. Absciscic acid triggers the endocytosis of the Arabidopsis KAT1 K⁺ channel and its recycling to the plasma membrane. *Curr Biol.* 2007;17(16):1396–402. <https://doi.org/10.1016/j.cub.2007.07.020>.
31. Chen YT, Liu H, Stone S, Callis J. ABA and the ubiquitin E3 ligase KEEP ON GOING affect proteolysis of the Arabidopsis KAT1 K⁺ channel and its recycling to the plasma membrane. *Plant J.* 2013;75(6):965–76. <https://doi.org/10.1111/tpj.12259>.
32. Kraft E, Stone SL, Ma L, Su N, Gao Y, Lau OS, et al. Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. *Plant Physiol.* 2005;139(4):1597–611. <https://doi.org/10.1104/pp.105.067983>.
33. Jue D, Sang X, Lu S, Dong C, Zhao Q, Chen H, et al. Genome-wide identification, phylogenetic and expression analyses of the ubiquitin-conjugating enzyme gene family in maize. *PLoS One.* 2015;10(11):e0143488. <https://doi.org/10.1371/journal.pone.0143488>.
34. Islam MS, Choudhury M, Majlish AN, Islam T, Ghosh A. Comprehensive genome-wide analysis of glutathione S-transferase gene family in potato (*Solanum tuberosum* L.) and their expression profiling in various anatomical tissues and perturbation conditions. *Gene.* 2018;639:149–62. <https://doi.org/10.1016/j.gene.2017.10.007>.
35. Lin Y, Zhang L, Zhang J, Zhang Y, Wang Y, Chen Q, et al. Identification of anthocyanins-related glutathione S-transferase (GST) genes in the genome of cultivated strawberry (*Fragaria × ananassa*). *Int J Mol Sci.* 2020;21(22):8708. <https://doi.org/10.3390/ijms21228708>.
36. Lin Y, Zhang J, Wu L, Zhang Y, Chen Q, Li M, et al. Genome-wide identification of GMP genes in Rosaceae and functional characterization of FaGMP4 in strawberry (*Fragaria × ananassa*). *Genes Genomics.* 2021;43(6):587–99. <https://doi.org/10.1007/s13258-021-01062-7>.
37. Zhang H, Jing X, Chen Y, Liu Z, Xin Y, Qiao Y. The genome-wide analysis of RALF-like genes in strawberry (wild and cultivated) and five other plant species (Rosaceae). *Genes.* 2020;11(2):174. <https://doi.org/10.3390/genes11020174>.
38. Li Q, Qiao X, Yin H, Zhou Y, Dong H, Qi K, et al. Unbiased subgenome evolution following a recent whole-genome duplication in pear (*Pyrus bretschneideri* Rehd.). *Hortic Res.* 2019;6:34. <https://doi.org/10.1038/s41438-018-0110-6>.
39. Hurst LD. The Ka/Ks ratio: diagnosing the form of sequence evolution. *Trends Genet.* 2002;18(9):486–7. [https://doi.org/10.1016/s0168-9525\(02\)02722-1](https://doi.org/10.1016/s0168-9525(02)02722-1).
40. Lynch M, Conery JS. The evolutionary fate and consequences of duplicate genes. *Science.* 2000;290(5494):1151–5. <https://doi.org/10.1126/science.290.5494.1151>.
41. Gray WM, Hellmann H, Dharmasiri S, Estelle M. Role of the Arabidopsis RING-H2 protein RBX1 in RUB modification and SCF function. *Plant Cell.* 2002;14(9):2137–44. <https://doi.org/10.1105/tpc.003178>.
42. Dharmasiri S, Dharmasiri N, Hellmann H, Estelle M. The RUB/Nedd8 conjugation pathway is required for early development in Arabidopsis. *EMBO J.* 2003;22(8):1762–70. <https://doi.org/10.1093/emboj/cdg190>.
43. Huang X, Tao Y, Gao J, Zhou X, Tang S, Deng C, et al. UBC9 coordinates inflammation affecting development of bladder cancer. *Sci Rep.* 2020;10(1):20670. <https://doi.org/10.1038/s41598-020-77623-9>.
44. Boulton SJ, Gartner A, Reboul J, Vaglio P, Dyson N, Hill DE, et al. Combined functional genomic maps of the *C. elegans* DNA damage response. *Science.* 2002;295(5552):127–31. <https://doi.org/10.1126/science.1065986>.
45. Jue D, Sang X, Shu B, Liu L, Wang Y, Jia Z, et al. Characterization and expression analysis of genes encoding ubiquitin conjugating domain-containing enzymes in *Carica papaya*. *PLoS One.* 2017;12(2):e0171357. <https://doi.org/10.1371/journal.pone.0171357>.
46. Gao Y, Wang Y, Xin H, Li S, Liang Z. Involvement of ubiquitin-conjugating enzyme (E2 gene family) in ripening process and response to cold and heat stress of *Vitis vinifera*. *Sci Rep.* 2017;7(1):13290. <https://doi.org/10.1038/s41598-017-13513-x>.
47. Cao Y, Meng D, Chen Y, Abdullah M, Jin Q, Lin Y, et al. Comparative and expression analysis of ubiquitin conjugating domain-containing genes in two *Pyrus* species. *Cells.* 2018;7(7):77. <https://doi.org/10.3390/cells7070077>.
48. Xie YG, Ma YY, Bi PP, Wei W, Liu J, Hu Y, et al. Transcription factor FvTCP9 promotes strawberry fruit ripening by regulating the biosynthesis of abscisic acid and anthocyanins. *Plant Physiol Biochem.* 2020;146:374–83. <https://doi.org/10.1016/j.plaphy.2019.11.004>.
49. Jung S, Lee T, Cheng CH, Buble K, Zheng P, Yu J, et al. 15 years of GDR: new data and functionality in the genome database for Rosaceae. *Nucleic Acids Res.* 2019;47(D1):D1137–45. <https://doi.org/10.1093/nar/gky1000>.
50. Finn RD, Coghill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, et al. The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res.* 2016;44:D279–D85. <https://doi.org/10.1093/nar/gkv1344>.
51. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, et al. CDD: a conserved domain database for the functional annotation of proteins. *Nucleic Acids Res.* 2011;39:D225–9. <https://doi.org/10.1093/nar/gkq1189>.
52. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol.* 2016;33(7):1870–4. <https://doi.org/10.1093/molbev/msw054>.
53. Zhang Z, Li J, Zhao XQ, Wang J, Wong GK, Yu J. KaKs_calculator: calculating Ka and Ks through model selection and model averaging. *Genomics Proteomics Bioinformatics.* 2006;4(4):259–63. [https://doi.org/10.1016/S1672-0229\(07\)60007-2](https://doi.org/10.1016/S1672-0229(07)60007-2).
54. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(ΔΔC_T)} method. *Methods.* 2001;25:402–8. <https://doi.org/10.1006/meth.2001.1262>.
55. Spolaore S, Trainotti, Casadoro G. A simple protocol for transient gene expression in ripe fleshy fruit mediated by *Agrobacterium*. *J Exp Bot.* 2001;52(357):845–50. <https://doi.org/10.1093/jexbot/52.357.845>.
56. Liu R, Zhang M, Li X. Comparisons of extraction solvents and quantitative methods of analysis of anthocyanins in strawberry and blueberry fruits. *Acta Horti Sin.* 2008;35:655–60. <https://doi.org/10.3724/SPJ.1005.2008.01083>.

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